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Identification of a *nodD*-Dependent Locus in the *Rhizobium* Strain NGR234 Activated by Phenolic Factors Secreted by Soybeans and Other Legumes

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Transfer of the strain NGR234 *nodD* 1 gene into the narrow host range *R. trifolii* strain ANU843 on either a 6.7-kb *Hind*III or 17-kb *Xho*I fragment broadens the host range of this bacterium to include the tropical legumes *Vigna unguiculata*, *Glycine ussuriensis*, *Leucaena leucocephala*, and siratro (*Macroptilium atropurpureum*). Contrary to previous data (Bassam *et al.* 1986), mutagenesis of the 17-kb *Xho*I fragment with a mini-Mu *lac* transposon (Mu dII1734) showed that a functional *nodD* 1 gene was essential for extended host range. Gene expression studies using both Mu dII1734 fusions and a promoter-cloning vector indicated that several loci, including the *nodD* 1 gene, are constitutively expressed. No evidence was found for regulation of the strain NGR234 *nodD* 1 gene by its product. Another locus, *nod*-81, was induced only in the presence of exudates from various plant species, including soybean (*Glycine max*). Whereas the expression of *nod*-81 was dependent on the presence of a functional *nodD* 1 gene product, a regulatory *nod*-box DNA sequence was not detected 5′ to this gene by using available oligonucleotide hybridization probes. The *nod*-81 locus was induced by genistein, daidzein, naringenin, and coumestrol from both cotyledon and root tissue of freshly germinated soybean seedlings. A broad spectrum of commercially available phenolic compounds stimulated induction of the *nod*-81 locus, including some that antagonize *nod* gene induction in other *Rhizobium* species. The *nodD* 1 gene product from strain NGR234 was shown to determine the spectrum of compounds that induce *nod*-81 expression.

Additional keywords: flavonoid inducer molecules, phenylpropanoid intermediates.

The fast-growing *Rhizobium* strain NGR234 (Trinick 1980) can nodulate a wide variety of plants, including tropical and temperate legumes, as well as the nonlegume tree *Parasponia andersonii* (Trinick and Galbraith 1980). It has a large Sym plasmid that encodes genes involved in nodulation (*nod*), nitrogen fixation (*nif*), and host-specific nodulation (*hsn*) (Morrison *et al.* 1983, 1984). Determinants of *hsn* are located on a 6.7-kb *Hind*III fragment from the Sym plasmid, whereas others occur elsewhere on the Sym plasmid (Bassam *et al.* 1986; Nayudu and Rolfe 1987). Recently, the *nodD* 1 gene has been implicated in the breadth of host specific nodulation ability of the strain NGR234 derivative strain MPIK3030 (Bachem *et al.* 1986; Horvath *et al.* 1987). In other rhizobia, the *nodD* gene product has a regulatory role and may contribute to host range specificity by its interaction with flavonoid compounds (Spaink *et al.* 1987b), which are secreted by the roots of legumes (Innes *et al.* 1985; Djordjevic *et al.* 1987; Rossen *et al.* 1985; Shearman *et al.* 1986; Mulligan and Long 1985; Horvath *et al.* 1987).

In *R. trifolii* and *R. leguminosarum*, certain flavonoids act as co-inducers of the expression of nodulation and host specific nodulation genes in rhizobia, whereas coumarins, flavanols, and isoflavones antagonize *nod* gene induction by inducers (Redmond *et al.* 1986; Firmin *et al.* 1986; Djordjevic *et al.* 1987). The *nodD* gene product is required for the flavone-dependent expression of *nod* genes in several *Rhizobium* species (Mulligan and Long 1985; Rossen *et al.* 1985). A conserved and possibly regulatory DNA sequence, called a *nod*-box, occurs 5′ to known inducible *nod* gene operons (Rostas *et al.* 1986; Schofield and Watson 1986).

To study gene expression in the region of the *nodD* 1 gene of strain NGR234, we inserted several Mu dII1734 *lac* transposons into the *Xho*I DNA fragment, which contains the 6.7-kb *Hind*III *hsn* fragment. Additional analysis of this region was done by fusing the *nodD* 1 promoter and an identified plant-factor dependent promoter to the promoterless *lacZ* gene of the plasmid vector pMP220.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1.

Nodulation tests. Seeds were sterilized and germinated by the methods of Cen *et al.* (1982) and Nayudu and Rolfe (1987). Siratro nodulation assays were done by using the plate assay of Rolfe *et al.* (1980). *Leucaena leucocephala* was assayed for nodulation on agar slopes in 120-ml sample jars (Bunzl Laboratory Products) by the method of Chen *et al.* (1985). Other plant species were assayed by using Magenta jars (Nayudu and Rolfe 1987), a variation of the Leonard jar assay system devised by E. Appelbaum (personal communication). Plant growth conditions were as described by Nayudu and Rolfe (1987).

Media. Trifolii-minimal medium, tryptone-yeast extract medium, Luria-Bertani (LB), and Fåhråeus media have been described previously (Rolfe *et al.* 1980) and Herridge's medium (Herridge 1977) was used for the cultivation of tropical legumes (full strength in agar and half strength in Magenta jar assays).

Plasmid transfers. Plasmids were transferred by using a

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triparental patch mating technique between donor, recipient, and the *Escherichia coli* strain HB101 carrying the helper-mobilizing plasmid pRK2013 (Figurski and Helinski 1979).

Recombinant DNA techniques. DNA isolations, visualization, hybridization, and ligations were done according to the methods of Maniatis *et al.* (1982). Hybond nylon membranes were used for DNA transfers (Amersham handbook 1985). Synthetic oligonucleotides were end-labeled with T4 polynucleotide kinase with γ-P^{32} ATP. The synthetic oligonucleotides used were complementary to the ANU289 *nod*-box -TGGTAAAATCGATTGTTTCG- (Scott 1986) and also to the MPIK3030 *nod*-box -TTGCATAATTGATCGTTCGG- (Horvath *et al.* 1987).

Mutagenesis with Mu dII1734. Plasmid pNGRX6, which carried a 17-kb *Xho*I fragment encoding *nodD* 1 and *hsn* determinants from strain NGR234, was mutagenised with the Mu dII1734 transposon (Castilho *et al.* 1984). Upon insertion, Mu dII1734 can create translational fusions of the promoterless *lac* genes with the target gene. Plasmid pNGRX6 was transformed into the *E. coli* strain POII1734, which harbors the Mu dII1734 (Castilho *et al.* 1984) and a temperature-sensitive helper Mu in its chromosome. *E. coli* strain POII1734 (pNGRX6) was freshly grown to log-phase in liquid LB medium containing 10 mM $MgCl_2$, and 1 ml of cells was incubated for 30 min at 43° C. The Mu dII1734 was induced to transpose by the helper Mu phage.

Fresh Mu particles were isolated after transferring the cells to 37° C for 2 hr. Two drops of chloroform were added, and the cells were gently vortexed to aid lysis. Cell debris was removed by spinning in a Eppendorf centrifuge for 1 min. Mu particles were used to tranfect 1 ml of fresh stationary phase *E. coli* strain PK1704 (Δlac) cells grown in LB containing 5 mM $MgSO_4$ and 10 mM $CaCl_2$. After preabsorption of the phage (28° C, 20 min, without shaking), the cells were diluted into 10 ml of liquid LB medium, shaken at 28° C for 45 min (to allow expression of antibiotic resistance markers), and plated onto LB agar medium containing 0.4 mg/ml X-Gal, chloramphenicol, and kanamycin for co-selection of the Cm^R marker of pNGRX6 and the Km^R marker of Mu dII1734. An indication of an in-phase fusion of Mu dII1734 to a promoter from pNGRX6 was detected by the blue coloration of individual *E. coli* colonies on X-Gal plates.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
NGR234	Wild-type, broad host range, fast-growing cowpea *Rhizobium*	Trinick 1980
ANU265	Sm^R, Sp^R, Nod^-, Sym plasmid cured strain NGR234 derivative	Morrison *et al.* 1983
ANU843	Wild-type *R. trifolii* strain	Djordjevic *et al.* 1983
RR1	*E. coli* strain used for the maintenance of plasmids (Sm^R)	Bolivar *et al.* 1977
POII1734	MC1040 (Mu *c*ts) with Mu dII1734 *lac*$^+$	Castilho *et al.* 1984
PK1704	*E. coli*, Δ(*lac*) 3*trp-49*	*Coli* genetic stock center, strain 5948
pRK2013	Narrow host range helper plasmid, has *tra* genes and ori-T of *Inc* P1 plasmid RK2 cloned into a *Col* E1 derivative and cannot be maintained in *Rhizobium*	Figurski and Helinski 1979
pNGRH6	6.7-kb *Hind*III fragment encoding *nodD*1 from strain NGR234 cloned into pSUP106	Bassam *et al.* 1986
pNGRX6	17-kb *Xho*I fragment encoding *nodD* 1 from strain NGR234 cloned into pSUP106	Bassam *et al.* 1986
pSUP106	Broad host range, *Inc* Q vector stably maintained in *Rhizobium*, Tc^R, Cm^R	Simon *et al.* 1983
pMP220	Broad host range, *Inc* P promoter cloning vector stably maintained in *Rhizobium*. Tc^R contains a promoterless *E. coli lacZ* gene	Spaink *et al.* 1987a

Assays of gene induction and expression. *In vivo* assays showing expression of Mu dII1734 gene fusions in bacteria near plant roots were done by using 0.16 mg/ml of X-Gal to assay for β-galactosidase activity (indicated by blue staining in the agar), as described by Redmond *et al.* (1986). Paper disks were wetted with 10 μl of 1×10^{-3} M ethanol solution of flavonoid, and then dried. β-galactosidase activity of bacteria grown in liquid medium was measured by using the method of Miller (1972) with slight modifications (Djordjevic *et al.* 1987).

Promoter cloning with pMP220. A 2.6-kb *Bam*HI-*Eco*RI DNA fragment and a 6-kb *Bam*HI DNA fragment were subcloned (see Fig. 1) into the promoter cloning vector pMP220 (Spaink *et al.* 1987a). Gene expression from those fragments was determined by measuring β-galactosidase activity by using O-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate as described elsewhere (Djordjevic *et al.* 1987).

Isolation and identification of phenolic compounds from soybean plants. Soybean seedlings (cv. Bragg) were axenically germinated on damp vermiculite and grown for 4 days. The roots and cotyledons of these soybean seedlings were blended to give a thin paste, which was allowed to stand at room temperature for 2–3 hr. An equal volume of methanol was added by stirring, and the mixture was allowed to stand overnight. The mixture was then centrifuged and the supernatant removed. The pellet was redispersed in 50% methanol and recentrifuged. The pellet was redispersed in 100% methanol, allowed to stand overnight, and then centrifuged again. The supernatants were then pooled, cleared by centrifugation, and carefully evaporated almost to dryness under reduced pressure. The residues were redispersed in 1 ml of 2 M trifluoroacetic acid at 100° C for 2 hr to liberate flavonoids from their glycosides. The mixture was extracted with dichloromethane and analyzed by HPLC.

For HPLC analysis, samples were dissolved in 20% aqueous methanol and separated on a Brownlee cartridge column (100 × 4.6 mm) with an RP-8 Spheri-5 packing. Elution was at 1 ml/min by using a 20–80% solvent gradient formed with a 0.2% aqueous trifluoroacetic acid and methanol for 30 min.

Chemicals. 7,4′-dihydroxyflavanone and 7,4′-dihydroxyflavone were prepared as before (Djordjevic *et al.* 1987). The following flavonoids were used: taxifolin, naringenin, morin, kaempferol, apigenin, 5,7-dihydroxy-4′-methoxy isoflavone (biochanin A), hesperitin, and umbelliferone (Sigma Chemical Co., St. Louis, MO); formononetin, daidzein, and genistein (ICN Biomedicals, Plainview, NY); luteolin (Research Plus, New York, NY); and coumestrol (Kodak).

RESULTS

Selection of Mu dII1734 insertions. Mu dII1734 insertions into plasmid pNGRX6 were selected in *E. coli* by plating the Mu lysogen onto LB agar plates containing chloramphenicol and kanamycin. The frequency of insertion of Mu dII1734 into plasmid pNGRX6 was about 10^{-4} per cell transfected with the lysogen. To enrich for

in-phase fusions to genes located on plasmid pNGRX6, *E. coli* colonies harboring mutated plasmids were screened for β-galactosidase activity by plating cells onto media containing X-Gal. About 80% of the colonies tested exhibited various levels of blue coloration. Some colonies turned blue after 24–36 hr, whereas others required up to 5 days. Seventeen Mu dII1734 insertions were mapped to the *Rhizobium* DNA of plasmid pNGRX6 and were selected for further analysis. The insertion positions are shown in Figure 1. A significant clustering of mutations to one side of the 17-kb fragment left free of insertions a 3.5-kb region between mutations 193 and 81 and a 2-kb region between mutations 45 and 85. In some cases, deletions of pNGRX6 DNA had occurred. Unlike deletions induced by Mu dI 1734 (Innes *et al.* 1985), no deletions originated from the ends of the Mu dII1734 DNA.

Broad host range nodulation ability. Mobilization of either the 17-kb *Xho*I fragment or its internal 6.7-kb *Hind*III fragment from strain NGR234 into *R. trifolii* strain ANU843 is known to extend its host range ability. We previously found that these transconjugants specifically nodulate siratro (*M. atropurpureum*) but not *Desmodium intortum*, *D. uncinatum*, *Lablab purpureus*, or *G. max* (Bassam *et al.* 1986). Further analysis has shown that transfer to *R. trifolii* of the 6.7-kb *Hind*III or the 17-kb *Xho*I fragments from the strain NGR234 Sym plasmid (on plasmid pNGRH6 or pNGRX6, respectively) also confers the ability to nodulate *Vigna unguiculata*, *Glycine ussuriensis*, and *Leucaena leucocephala*. Strain NGR234 or ANU843 carrying the 17-kb *Xho*I fragment induced two to three nodules per plant on *G. ussuriensis* and *L. leucocephala* and 25 nodules per plant on *V. unguiculata* after 20 days and the onset of nodulation occurred at the same time. In contrast, *R. trifolii* strain ANU843 carrying

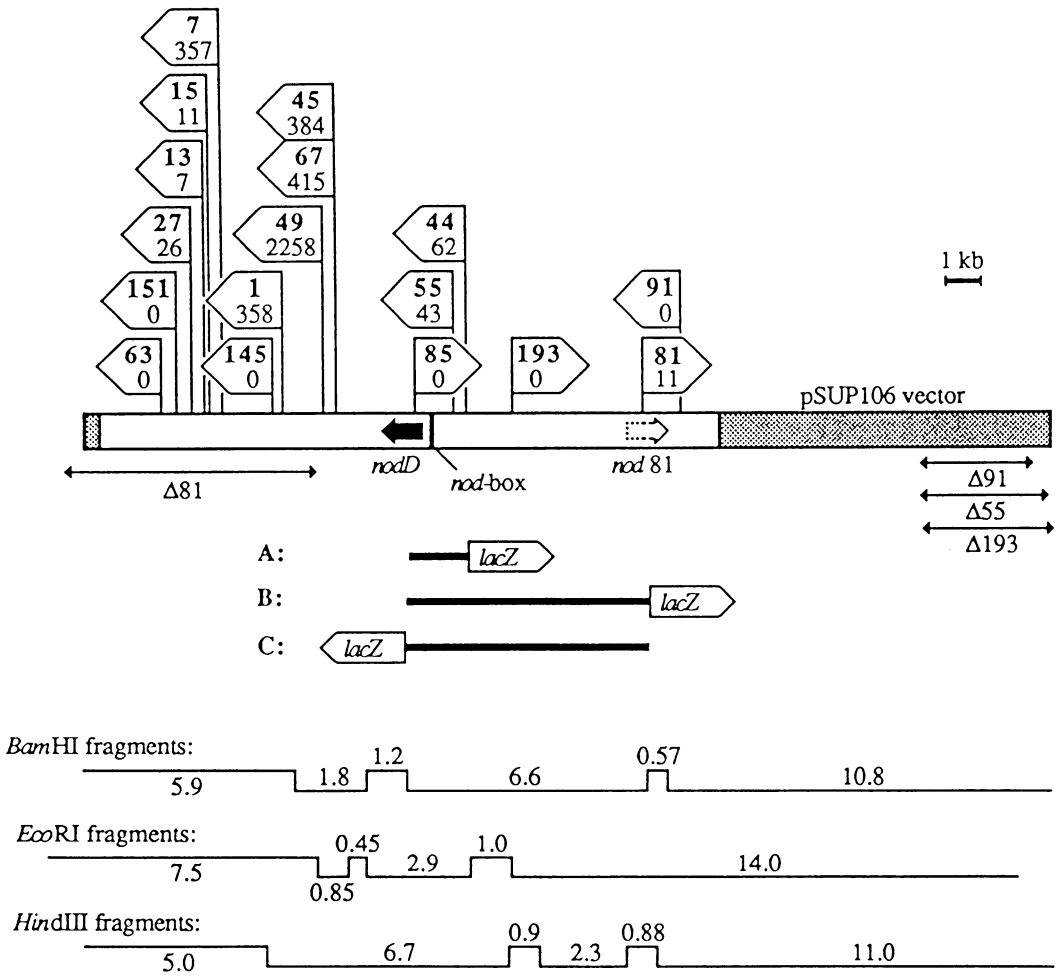

Fig. 1. Constructs used to monitor gene expression in the 17-kb *Xho*I fragment of strain NGR234. The plasmid pNGRX6 is illustrated, in which the 17-kb *Xho*I fragment is shown as an open rectangle and vector sequences are filled with grey hatching. *Bam*HI, *Hind*III, and *Eco*RI restriction fragments for plasmid pNGRX6 are also shown. The insertion sites for Mu dII1734 in plasmid pNGRX6 are indicated by the open flags (which show the inferred direction of transcription). The mutation numbers appear uppermost in the open flags (in bold face), and the numbers beneath indicate the level of β-galactosidase activity obtained from each fusion in strain NGR234 without addition of inducer molecules. The extent of deletions (Δ) that occurs for some fusions are shown as double-headed arrows. The location of the *nodD* 1 gene and its direction of transcription is shown (black arrow). The *nod*-81 gene is illustrated as a broken arrow (because the size of this gene is not known). The location of the *nod*-box-like sequence is shown as a vertical bar. **A,** the 1.8-kb *Eco*RI-*Bam*HI fragment subcloned in pBB18 to measure expression from the presumptive *nod*-box locus, its position in relation to the 17-kb *Xho*I fragment, and the location of the *lacZ* fusion in this construction. **B,** the 6.6-kb *Bam*HI fragment subcloned in pBB81 to measure expression of the *nod*-81 gene. **C,** the 6.6-kb *Bam*HI fragment subcloned in the reverse orientation in PBBD1 to measure expression of the *nodD* 1 gene from strain NGR234.

the 6.7 kb *Hind*III fragment nodulated each plant species with a significant delay (varying between 2–5 days), although the number of nodules induced was eventually the same. Strain ANU843 alone induced small, white root swellings on all three plants species. However, these swellings contrasted with nodules produced by *R. trifolii* strains carrying strain NGR234 DNA fragments, which were large, well developed, and superficially indistinguishable from those produced by strain NGR234. Bacteria isolated from each plant species retained the antibiotic resistance markers of the original inoculants.

Mutagenesis of the strain NGR234 17-kb *Xho*I fragment. Each of the Mu dII1734-mutated pNGRX6 plasmids was transferred to *R. trifolii* strain ANU843 and the transconjugants tested for extended host range to *V. unguiculata*, *G. ussuriensis*, and *L. leucocephala*. All but one of the pNGRX6 derivatives (plasmid pNGRX6::85; shown in Fig. 1) were capable of extending the host range ability to each plant species. The mutation in plasmid pNGRX6::85 maps in the *nodD* 1 gene of strain NGR234, and its phenotype shows that the *nodD* 1 gene is required for extension of host range in *R. trifolii*.

R. trifolii carrying mutant plasmid pNGRX6::81 was slower to form nodules on test legumes than the same strain carrying the 17-kb *Xho*I fragment, and it gave a nodulation response that was very similar to that of strain ANU843 carrying the 6.7-kb *Hind*III fragment. *R. trifolii* strain ANU843 (pNGRX8::81) is not restricted in its ability to nodulate *V. unguiculata*, *G. ussuriensis*, or *Macroptilium atropurpureum* by using the Magenta jar assay, but when compared with ANU843 carrying pNGRX6, nodulation was delayed by several days on *L. leucocephala* (tested on agar slopes) and *M. atropurpureum* (tested on agar plates). The mutation of this gene may have a subtle phenotype and may therefore be important for rapidity of nodulation or host specificity. Plasmid pNGRX6::81 has an insertion of Mu dII1734 that maps outside the internal 6.7-kb *Hind*III fragment (of plasmid pNGRH6), as well as a deletion of about 7 kb (Fig. 1). The deletion does not extend from the site of the Mu dII1734 fusion, but from within the vector to a site within the *Hind*III fragment, leaving the *nodD* 1 gene and most of the internal 6.7-kb *Hind*III fragment intact. Other pNGRX6 plasmids with Mu dII1734 insertions in the region deleted in pNGRX6::81 did not show a similarly delayed nodulation phenotype, indicating that the deletion does not contribute to the delayed nodulation phenotype.

Expression of Mu dII1734 gene fusions in *Rhizobium*. Whereas several fusions expressed β-galactosidase activity at high constitutive levels (Fig. 1), nodulation tests using *R. trifolii* strain ANU843 carrying these plasmids did not identify a nodulation-defective phenotype for these insertion mutants. To test for genes dependent on plant-secreted factors for expression, seedlings of 12 different legume species (including *G. max*) were incubated in the dark at 28° C on X-Gal plates (20 μg per plate) with overlays of early log-phase cells of strain ANU265 containing various pNGRX6 plasmids with Mu dII1734 insertions. Only bacteria containing plasmid pNGRX6::81 showed a significant induction of β-galactosidase activity, and this occurred only in those *Rhizobium* cells in the immediate vicinity of the legume seedlings. Strain ANU265 (pNGRX6::81) responded best to the presence of *G. max* seedlings in which the bacteria in the soft agar overlay around each seedling turned blue after 24–36 hr of incubation. A pale blue region surrounded the seedling (with a radius of about 12 mm), with a much darker halo further out (with a radius of about 34 mm). Early log-phase *Rhizobium* cells incorporated into the soft agar overlay were found to express more β-galactosidase activity than late log or stationary phase cells in the presence of seedlings.

Chromatographic analysis of soybean extracts. Extracts prepared from cotyledon and root tissue showed that inducing compounds were present and could, when incubated with *Rhizobium* cells containing pNGRX6::81, stimulate significant expression of β-galactosidase activity. Higher amounts of net stimulatory activity were found in cotyledon tissue. HPLC analysis of root extracts revealed naringenin as the main flavonoid component, with smaller amounts of daidzein, genistein, coumestrol, and a number of minor uncharacterized compounds with long elution times. Increased amounts of the same components were obtained by acid hydrolysis of water-soluble glycosides. Extracts of cotyledons contained the same flavonoids, with the exception that much higher amounts of naringenin were present.

Transcription of pNGRX6::81. Strain ANU265 containing plasmid pNGRX6::81 was tested for induction by a wide range of flavonoids and related compounds. Paper disks impregnated with each test compound were applied to a bacterial lawn of early log-phase cells on minimal medium plates containing X-Gal. A range of flavonoids (including those listed in Materials and Methods) was able to induce *lac* expression from pNGRX6::81, but the degree of induction was weaker than that observed for similarly prepared lawns of *R. trifolii* containing *lacZ* fusions to the *nodA* gene (Redmond *et al.* 1986).

Quantitative measurements of the expression of the pNGRX6::81 fusion were made by assaying β-galactosidase activity in bacteria grown in solution using ONPG as substrate. Four-hr exposures to the signal compounds were sufficient to detect induction; however, 16-hr inductions were used routinely, as this gave more reliable results with the less active compounds. Concentrations as low as 5×10^{-7} M were sufficient to induce expression of the pNGRX6::81 *lacZ* fusion with each test compound.

Expression of pNGRX6::81 in either strain NGR234 or ANU265 was induced by a variety of flavonoids (Fig. 2), including those that induce *R. trifolii* and *R. meliloti* nodulation genes (7,4′-dihydroxyflavone, apigenin, and luteolin), as well as others that do not (taxifolin, kaempferol, and morin). Plasmid pNGRX6::81 was also induced by the following: two anti-inducers of *R. trifolii nod* gene induction; umbelliferone (7-hydroxycoumarin), and formononetin (7-hydroxy,4′-methoxyisoflavone); by the flavanones 7,4′-dihydroxyflavanone, naringenin (7,5,4′-trihydroxyflavanone), and hesperitin (3′, 5,7-trihydroxy-4′-methoxyflavanone); by the isoflavones daidzein (7,4′-dihydroxyisoflavone), genistein, and biochanin A; and by the coumestan coumestrol (3,9-dihydrocoumestan). Induction levels achieved in strain ANU843 were similar to those obtained in NGR234 except with compounds that are strong co-inducers of *R. trifolii nod* gene expression.

Induction by flavonoid compounds. To characterize further the gene at the locus defined by the pNGRX6::81 mutation, a 6.6-kb *Bam*HI fragment (from within the 17-kb *Xho*I fragment) was subcloned into the promoter cloning vector pMP220. One end of this fragment comes to within about 300 bp of the insertion site of the Mu dII1734::81 mutation and, at the other end, bisects the *nodD* 1 gene (Fig. 1). By subcloning this fragment in both orientations into plasmid pMP220, we were able to monitor expression of both the locus assayed in pNGRX6::81 (with pBB81) and

the *nodD* 1 gene (with pBBD1).

Plasmid pBB81 was conjugated into strains NGR234, ANU1255 (a *nodD* 1::Tn*5* mutant of strain NGR234), ANU265 (Sym⁻ NGR234), *R. trifolii* strain ANU843, ANU851 (a *nodD*::Tn*5* mutant of strain ANU843), and ANU845 (a Sym⁻ strain ANU843), and then tested for response to flavonoid compounds (Table 2). In strain NGR234, the spectrum of flavonoids affecting the inducible promoter of plasmid pBB81 was identical to that of plasmid pNGRX6::81. However, the level of induction to each compound tested was three- to fourfold lower with pBB81. This was possibly due to copy-number differences between the two plasmids used. When introduced into *R. trifolii* strain ANU843, pBB81 showed induced expression to only those compounds known to induce expression of the *R. trifolii* nodulation genes. Compounds possessing activity in the presence of the NGR234 *nodD* had no activity in ANU843. In genetic backgrounds lacking a *nodD* gene (ANU1255, ANU265, ANU851, or ANU845), no significant induction was observed to any signal compound tested when introduced into strain. Thus, the locus assayed in pNGRX6::81 specifically requires a functional *nodD* 1 gene for induction by plant-derived signal compounds. Because expression of the locus assayed in pNGRX6::81 is *nodD*-dependent, and this mutation results in a delayed nodulation phenotype, we tentatively designated this locus *nod*-81.

***nod*-box sequence on the *Xho*I fragment.** The 17-kb *Xho*I fragment was probed with two synthetic oligonucleotides specific for *nod*-box sequences present in strains MPIK3030 (Horvath *et al.* 1987) and ANU289 (Scott 1986; see Materials and Methods). Only one *nod*-box sequence was detected on the 17-kb *Xho*I fragment under the conditions used by hybridization to the strain MPIK3030-specific probe (data not shown). No hybridization was detected to the strain ANU289-specific probe. The hybridizing region was at a site 5′ to the *nodD* 1 gene of strain NGR234 (see Fig. 1).

To determine if the *nod*-81 gene was transcribed from the single *nod*-box sequence on the 17-kb *Xho*I fragment from strain NGR234, a 1.8-kb *Bam*HI-*Eco*RI fragment was subcloned into the expression vector pMP220 to form plasmid pBB18 (Fig. 1). The mapped site of the *nod*-box sequence is within 1 kb of the *Eco*RI end of this fragment. Plasmid pBB18 should yield inducible β-galactosidase activity if the *nod*-box sequence 5′ to the *nodD* 1 gene was the promoter site of the *nod*-81 gene.

Upon introduction of plasmid pBB18 into strains NGR234, ANU1255, ANU265, and *R. trifolii* strains ANU843, ANU851, and ANU845, only low levels of constitutive β-galactosidase activity were detected in all six strains (about 200 units of β-galactosidase activity in strain NGR234 derivatives and about 150 units in *R. trifolii*). Addition of signal compounds did not affect the level of β-galactosidase activity obtained in each *Rhizobium* strain, indicating that the *nod*-box sequence immediately 5′ to *nodD* 1 is unlikely to be the promoter for the *nod*-81 gene.

The expression of the strain NGR234 *nodD* 1 gene. To assay expression of the strain NGR234 *nodD* 1 gene, plasmid pBBD1 was conjugated into strains NGR234, ANU1255, ANU265, and *R. trifolii* strains ANU843, ANU851, and ANU845. The level of transcription from the *nodD* 1 promoter was the same in each NGR234 derivative strain (about 200 units) and was unaffected by the addition of flavonoids. This indicates that the *nodD* 1 gene is

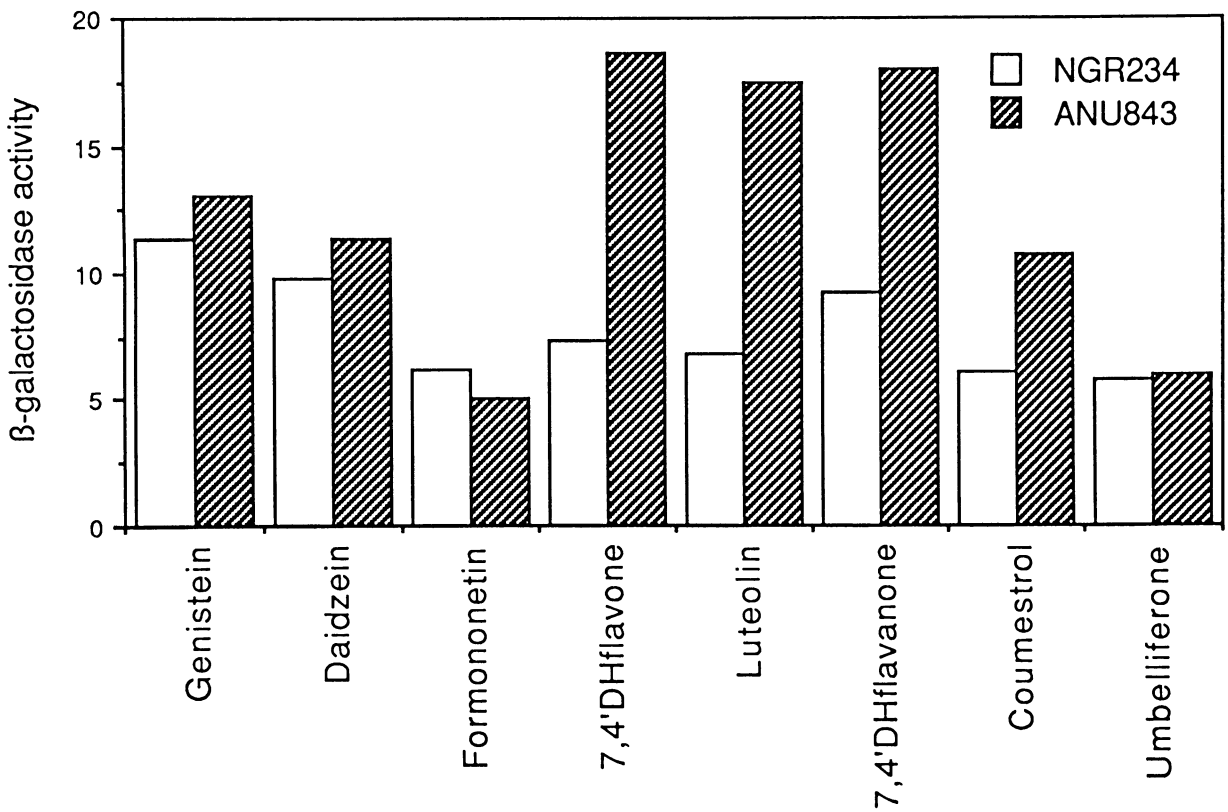

Fig. 2. Induction of mutant plasmid pNGRX6::81 by various flavonoid compounds in the cellular environment of either strain NGR234 or *R. trifolii* strain ANU843. The level of induction is expressed as a multiplication of the background level of β-galactosidase activity that was about 18 units in strain NGR234 and about 13 units in *R. trifolii*. Assays were done using an 16-hr induction to 5×10^{-7} M concentration of each compound, as described in Materials and Methods. Values shown are optimal for more than five repeats with each compound. Standard deviations varied between 5–25 units for each assay. DH = dihydroxy.

constitutively expressed and that NodD does not autoregulate *nodD* 1 expression in strain NGR234. Additional experiments measured expression of the *nodD* 1 gene over the growth cycle of the bacterium by using plasmid pBBD1 in strains NGR234, ANU1255, and ANU265 (grown in liquid Bergersen's Modified Medium, with shaking, at 30° C) (Rolfe *et al.* 1980). Expression of the *nodD* 1 gene remained constant at about 200 units throughout the growth cycle of each strain.

The *nodD* 1 promoter was constitutively transcribed at a high level in strains ANU851 and ANU845 (to 1,100 units of activity). In strain ANU843, however, the level was consistently reduced from about 1,100 units to about 900 units, suggesting that the *R. trifolii* NodD can regulate expression of the strain NGR234 *nodD* 1 gene.

DISCUSSION

The broad host range properties of strain NGR234 can be partly explained by the characteristics of its single functional *nodD* gene. Transfer of this gene into the narrow host range *R. trifolii* strain ANU843 on either a 6.7-kb *Hind*III or a 17-kb *Xho*I fragment extends the host range of this bacterium to include several legume species. Contrary to previous results (Bassam *et al.* 1986), mutagenesis showed that the *nodD* 1 gene from strain NGR234 is essential to broaden the host range phenotype of *R. trifolii* recipients to include several legume species. Further investigation of the original mutants obtained by Bassam *et al.* (1986) has revealed that an error was made in the mapping of several Tn*5* insertions initially located in *nodD*. Several Mu dII1734 insertions into plasmid pNGRX6 (::49, ::67, ::45, ::55, ::44; Fig. 1) were found to occur in the presumptive *hsn* loci identified on the 6.7-kb *Hind*III fragment by Bassam *et al.* (1986). However, when pNGRX6 plasmids carrying these mutations were tested in *R. trifolii*, they did not affect the extended host range phenotype seen with pNGRX6 transconjugants in *R. trifolii*. The mutations described here were assayed in a multicopy vector, whereas the original mutants were obtained by chromosomal integration of the 6.7-kb *Hind*III fragment. Copy number may thus be important in the identification of a nodulation phenotype.

The extended host range phenotype conferred by the 17-kb *Xho*I fragment does not include all plants normally nodulated by strain NGR234 (e.g., *Desmodium intortum, D. uncinatum, L. purpureus, G. max, G. soja*, or *G. wightii*) (Bassam *et al.* 1986), indicating that other *hsn* loci exist in strain NGR234. This is consistent with the report of Nayudu and Rolfe (1987), who showed host specificity loci in at least two other regions of the strain NGR234 Sym plasmid.

The application of a new mutagenesis procedure in *E. coli* generated a series of Mu dII1734 insertions in the 17-kb *Xho*I fragment. This allowed the location and direction of transcription, expression, and phenotype of genes on this fragment to be determined in *Rhizobium*. These fusions identified several loci on the *Xho*I fragment that are constitutively expressed and one that is induced by exudates from the roots of several legumes, including soybean.

Only mutations located in *nodD* (*nod*-85) and at the *nod*-81 locus affected the nodulation phenotype of strains containing derivatives of pNGRX6. Because the *nod*-85 mutation is located in *nodD* in the opposite orientation to the *nodD* transcript (M. Nayudu, personal communication), this explains the lack of β-galactosidase activity obtained from this fusion. Mutation at the *nod*-81 locus affects the rapidity of nodulation, and this mutant has a similar delayed-nodulation phenotype to *R. trifolii* transconjugant strains carrying the 6.7-kb *Hind*III. Because a deletion has also occurred in the construction of the pNGRX6::81 derivative, we cannot discount that this also contributes to the phenotype imparted by this plasmid.

We have shown that a broad spectrum of flavones and related compounds are able to induce expression of the *nod*-81 gene, some of which are anti-inducers of nodulation gene induction in *R. leguminosarum* and *R. trifolii* (Firmin *et al.* 1986; Djordjevic *et al.* 1987). In common with other examples of flavonoid-induced *nod* gene transcription (Rossen *et al.* 1985; Redmond *et al.* 1986; Djordjevic *et al.* 1987), the *nod*-81 gene requires a functional *nodD* gene product and displays zones of stronger and weaker induction around seedlings. This may be caused by release of compounds from seedlings that inhibit as well as stimulate expression of the *nod*-81 gene or, alternatively, the release of other plant factors that inhibit growth specifically in these areas. Each of the commercial flavonoid compounds tested did not significantly inhibit normal growth of any *Rhizobium* strain used during induction assays of up to 18 hr. This indicates that flavonoids are not toxic to the *Rhizobium* strains at concentrations of less than 1×10^{-6} M. The isoflavones daidzein and genistein and the flavanones naringenin and coumestrol (but not umbelliferone) were isolated from tissues of germinated soybean seedlings (cv. Bragg), suggesting that they may be the signal compounds from this plant. These results are in agreement with those of Porter *et al.* (1986) who found genistein,

Table 2. Inducibility of the *nod*-81 gene by various flavonoids in several *Rhizobium* strains

Compound	Strain harboring pBB81[a]					
	NGR234	ANU1255	ANU265	ANU843	ANU851	ANU845
None	59 ± 16	68 ± 13	70 ± 12	35 ± 4	35 ± 2	26 ± 4
7,4′-dihydroxyflavone	138 ± 24	66 ± 19	65 ± 15	169 ± 10	40 ± 4	35 ± 4
Apigenin	127 ± 6	63 ± 12	65 ± 15	53 ± 7	38 ± 8	32 ± 5
Kaempferol	115 ± 7	68 ± 12	59 ± 15	30 ± 6	30 ± 2	33 ± 9
Luteolin	103 ± 9	65 ± 12	59 ± 12	101 ± 18	29 ± 4	27 ± 6
7,4′-dihydroxyflavanone	118 ± 20	63 ± 15	63 ± 14	73 ± 19	41 ± 4	35 ± 3
Naringenin	144 ± 9	70 ± 14	N.D.	63 ± 14	33 ± 8	31 ± 5
Daidzein	141 ± 7	63 ± 12	56 ± 19	38 ± 16	35 ± 2	31 ± 5
Genistein	119 ± 3	76 ± 6	63 ± 4	24 ± 3	28 ± 5	22 ± 1
Formononetin	113 ± 13	66 ± 12	53 ± 8	25 ± 1	25 ± 3	23 ± 1
Biochanin A	98 ± 7	57 ± 12	52 ± 13	26 ± 5	26 ± 3	25 ± 3
Umbelliferone	82 ± 7	68 ± 15	54 ± 7	36 ± 4	33 ± 1	30 ± 2
Coumestrol	118 ± 5	73 ± 9	64 ± 15	42 ± 2	38 ± 7	29 ± 4

[a] Measurements are in units of β-galactosidase activity, following induction of the plasmid pBB81 (which carries the *nod*-81 gene promoter fused to a *lacZ* gene). Assays were done by using an overnight induction with 1×10^{-6} M of each compound, as described in Materials and Methods. Values shown are an average of three readings (± standard deviations), with two repetitions.

daidzein, and coumestrol in roots and kaempferol, naringenin, quercetin, genistein, and daidzein in leaves of soybean plants. These results also are in agreement with those of Kosslak *et al.* (1987), who identified daidzein and genistein as the signal compounds from soybean (cv. Williams) for the *Bradyrhizobium japonicum* strain USDA123. It is possible that use of such fusions may form the basis of a new general method for monitoring the amount or release of such plant metabolites.

We were unable to show that transcription of the *nod*-81 gene occurs from a *nod*-box sequence by hybridization analysis. The strain ANU289 *nod*-box failed to hybridize to the known *nod*-box sequence that occurs immediately 5′ to the NGR234 *nodD*, whereas the MPIK3030 *nod*-box hybridized only to the known *nod*-box. It seems likely, then, that if an additional *nod*-box sequence is found 5′ to the *nod*-81 locus, it will be sufficiently different to account for the failure to hybridize. Given that the *R. trifolii nodD* gene product can mediate the flavonoid-dependent expression of the *nod*-81 gene and that all *R. trifolii nodD*-dependent promoters (thus far defined) are preceded by a *nod*-box sequence, the presence of an additional *nod*-box 5′ to *nod*-81 seems likely. Furthermore, there is little doubt that the strain NGR234 *nodD* 1 gene can recognize promoters activated by the *R. trifolii* NodD. This is illustrated by the ability of the 6.7-kb *Hind*III fragment from strain NGR234 (which encodes the *nodD* 1 gene) to restore nodulation ability on clover plants to *R. trifolii nodD* mutants (complementation), as well as extend the host range of the recipient to siratro plants (Bassam *et al.* 1986). For this to occur, the *nodD* 1 gene must, as a minimum requirement, facilitate transcription from the promoter of the *R. trifolii nodABCIJ* operon.

As with other *nodD* dependent genes in other *Rhizobium* species (Spaink *et al.* 1987a; Horvath *et al.* 1987), expression of the *nod*-81 gene is specifically modulated by the *nodD* gene product of strain NGR234 and *R. trifolii* strain ANU843. Each NodD specifies a different pattern of inducer compounds. The range of inducer compounds specified by the strain NGR234 NodD 1 gene is very wide, whereas a narrow range of inducers are specified by the *R. trifolii nodD* gene product. This is not always the case, as Kosslak *et al.* (1987) showed that the introduction to *R. trifolii* of a DNA fragment of *B. japonicum* carrying both *nodD* and a *nodC:lacZ* fusion resulted in sensitivity to inducer compounds of the recipient strain only.

The strain NGR234 NodD was unable to regulate transcription of the *nodD* gene, thus illustrating similarity to the regulation of the *R. meliloti nodD* 1 gene (Mulligan and Long 1985). Thus, the regulation of the *nodD* gene of these strains differs from that in *R. trifolii* and *R. leguminosarum*, in which autoregulation of the expression of the *nodD* gene by NodD occurs (Rossen *et al.* 1985; J. McIver and M. Djordjevic, unpublished results). However, the *R. trifolii nodD* gene product is capable of regulating the expression of the NGR234 *nodD* gene and, therefore, autoregulation may be influenced by both the *nodD* gene product as well as the recognized target sequence. In *R. trifolii*, expression of the strain NGR234 *nodD* 1 gene was about fivefold higher than in the parent strain NGR234. The reason for this is not known.

Recently, a second *nodD*-like gene has been detected by DNA hybridization in strain MPIK3030 (Horvath *et al.* 1987). Our results show that this second *nodD* is not directly involved in nodulation for two reasons. First, a mutation in the *nodD* 1 gene results in the complete loss of nodulation ability on all tested host plants (Bassam *et al.* 1986). Second, a mutation in the *nodD* 1 gene also results in complete loss of induction ability of the *nod*-81 gene. Similarly, mutation in the single *nodD* gene of *R. trifolii* or *R. leguminosarum* also results in complete loss of both nodulation capacity and the ability to induce *nod* gene operons (Djordjevic *et al.* 1985; Rossen *et al.* 1985). It is clear from the results presented that strain NGR234 has employed a different evolutionary strategy with respect to host range ability, as the product of the single *nodD 1* gene is capable of interacting with a wide variety of stimulator compounds. The ability of the *nodD* gene from NGR234 to impart extended host range capacity to *R. trifolii* implies that this ability to interact with many inducer molecules is one of the chief determinants of broad host range ability.

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